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| <p>(54) Title: MODIFIED HEPATOCYTES AND USES THEREFOR</p>  |           |   |
| <p>The diagram illustrates four recombinant retrovirus vectors used for gene transfer. Each vector is represented as a linear sequence of DNA elements:</p> <ul style="list-style-type: none"> <li><b>LTR-LDLR:</b> Contains the LTR (Left Terminal Repeat), a splice donor (SD) site, the LDLR gene, and a polyA signal (represented by a hatched box).</li> <li><b>BA-LDLR:</b> Contains the LTR, an SD site, the gag gene, the BA (Bacterial Artificial Chromosome) gene, the LDLR gene, and a polyA signal.</li> <li><b>TK-LDLR:</b> Contains the LTR, an SD site, the gag gene, the TK (Thymidine Kinase) gene, the LDLR gene, and a polyA signal.</li> <li><b>H4-LDLR:</b> Contains the LTR, an SD site, the gag gene, the H4 (Histone H4) gene, the LDLR gene, and a polyA signal.</li> </ul> <p>A scale bar at the bottom indicates the length of the vectors in kilobases (Kb), ranging from 0 to 6 Kb.</p> |           |   |
| <p>(57) Abstract</p> <p>Genetically engineered or transduced hepatocytes which express genetic material of interest introduced or incorporated into them, as well as methods of producing, transplanting and using the genetically engineered hepatocytes. The genetic material of interest can be incorporated through use of a vector, such as a recombinant retrovirus, which contains the genetic material of interest, or by other means.</p>   |           |   |

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MODIFIED HEPATOCYTES AND USES THEREFORDescriptionBackground

05       The liver is of endodermal origin and the largest  
gland in the human body. It has numerous crucial roles,  
including bile secretion, participation in carbohydrate,  
lipid and protein metabolism, fibrinogen production and  
detoxification of drugs. The liver also serves as the  
main site at which nutrients absorbed from the gastro-  
10   intestinal tract and transported via the blood are  
processed for use by other body cells.

Hepatocytes, which are the main type of parenchymal  
or distinguishing cell in the liver, carry out the liver  
functions and, thus, are responsible for synthesizing,  
15   degrading and storing a wide variety of substances. In  
addition, a system of small channels (canaliculi) and  
larger ducts connects hepatocytes with the gut lumen.  
Through this route, hepatocytes secrete bile, an emulsi-  
fying agent which helps in absorption of ingested fats.  
20   Hepatocytes are also the main location at which lipo-  
protein particles for export are made; enzymes respons-  
ible for synthesis of the lipid constituents of lipo-  
proteins occur in hepatocyte membranes.

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Because of the many important functions the liver has, its inability to function normally (e.g., as a result of a genetic defect or damage caused by alcohol or other toxic substances) will often have significant adverse effects on an individual's health. A means by which normal function can be conferred upon or restored to a liver whose function is compromised would be very useful in treating, correcting or preventing such an abnormality.

#### 10 Summary of the Invention

The invention described herein relates to genetically engineered or transduced hepatocytes which express genetic material (DNA or RNA) of interest introduced or incorporated into them, as well as to methods of producing, transplanting and using the genetically engineered hepatocytes. The genetic material of interest can be incorporated through the use of a vector, such as a recombinant retrovirus, which contains the genetic material of interest, or by other means.

20 Hepatocytes of the present invention express the genetic material of interest. Such genetic material of interest can be: 1) genetic material present in and expressed at biologically effective levels by normal hepatocytes, but present in or expressed in less than normal quantities in the hepatocytes prior to transfer of genetic material of interest into them by the method of the present invention; 2) genetic material not present in normal hepatocytes; or 3) genetic material present in normal hepatocytes but not expressed at biologically effective levels in such cells, alone or in any combination thereof.

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In hepatocytes of the present invention, the genetic material of interest can be incorporated into the cellular genetic material (e.g., into genomic DNA) or can be present extrachromosomally (i.e., expressed episomally).

05 The genetic material of interest can be DNA or RNA; the DNA can constitute all or a portion of a gene of interest (i.e., one whose expression in hepatocytes is desired).

The genetic material incorporated into and expressed by hepatocytes of the present invention can additionally  
10 include genetic material (e.g., DNA) encoding a selectable marker, which provides a means by which cells expressing the genetic material of interest are identified and selected for. Hepatocytes containing incorporated genetic material (i.e., genetic material of  
15 interest and, optionally, genetic material encoding a selectable marker) are referred to as transduced hepatocytes.

Genetic material can be introduced into hepatocytes ex vivo or in vivo. That is, it can be introduced, by  
20 means of an appropriate vector, into isolated (cultured) hepatocytes, which are subsequently transplanted into the recipient. Alternatively, it can be introduced directly into the recipient in such a manner that it is directed to and taken up by target cells (hepatocytes), where it  
25 is incorporated and expressed. Particularly useful for this purpose are retroviral vectors which have an amphotropic host range and include the genetic material of interest which is to be incorporated into hepatocytes.

Retroviral vectors have been used to stably  
30 transduce hepatocytes with genetic material which included genetic material encoding a polypeptide or protein of interest and genetic material encoding a

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dominant selectable marker. Genetic material including DNA encoding a polypeptide of interest and DNA encoding a dominant selectable marker has been introduced into cultured hepatocytes. Expression of the genetic material by the hepatocytes into which they have been incorporated has also been demonstrated.

A method of transplanting transduced hepatocytes which express the incorporated genetic material they contain is also a subject of the present invention. Transduced hepatocytes of the present invention are used, for example, for the delivery of polypeptides or proteins which are useful in prevention and therapy of an acquired or an inherited defect in hepatocyte (liver) function. For example, they can be used to correct an inherited deficiency of the low density lipoprotein receptor (LDLR), which is synthesized in hepatocytes, and to correct an inherited deficiency of ornithine transcarbamylase (OTC), which results in congenital hyperammonemia.

Hepatocytes of the present invention are useful as a means by which abnormal hepatocyte function can be corrected. That is, hepatocytes can be transduced with genetic material of interest selected to compensate for over- or underproduction of a protein or peptide which is synthesized correctly, but in inappropriate amounts in the hepatocytes. Alternatively, they can be transduced with genetic material of interest encoding a protein or polypeptide which is produced in an appropriate quantity, but is functionally defective (e.g., because of an abnormal structure or amino acid makeup).

Hepatocytes to be modified ex vivo, as described herein, can be obtained from an individual, modified and

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returned to the individual by transplanting or grafting or, alternatively, can be obtained from a donor (i.e., a source other than the ultimate recipient), modified and applied to a recipient, again by transplanting or  
05 grafting.

An important advantage of the procedure of the present invention is that the genetically engineered hepatocytes can be used to provide a desired therapeutic protein or peptide by a means essentially the same as  
10 that by which the protein or peptide is normally produced and, in the case of autologous grafting, with little risk of an immune response and graft rejection. In addition, there is no need for extensive (and often costly) purification of a polypeptide before it is administered to an  
15 individual, as is generally necessary with an isolated polypeptide. Hepatocytes modified according to the present invention produce the polypeptide as it would normally be produced.

Because genes can be introduced into hepatocytes  
20 using a retroviral vector, they can be "on" (subject to the retroviral vector control; in such a case, the gene of interest is transcribed from a retroviral promoter. A promoter is a specific nucleotide sequence recognized by RNA polymerase molecules that start RNA synthesis.  
25 Alternatively, retroviral vectors having additional promoter elements (in addition to the promoter incorporated in the recombinant retrovirus), which are responsible for the transcription of the genetic material of interest, can be used. For example, a construct in  
30 which there is an additional promoter modulated by an external factor or cue can be used, making it possible to control the level of polypeptide being produced by the



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modified hepatocytes by activating that external factor or cue. For example, heat shock proteins are proteins encoded by genes in which the promoter is regulated by temperature. The promoter of the gene which encodes the metal-containing protein metallothioneine is responsive to cadmium ( $\text{Cd}^{++}$ ) ions. Incorporation of this promoter or another promoter influenced by external cues also makes it possible to regulate the production of the polypeptide by the engineered hepatocytes.

10 Brief Description of the Drawings

Figure 1 is a schematic representation of a wild type murine leukemia virus (retroviral) genome.

Figure 2 is a schematic representation of retroviral vectors, each having a recombinant genome, which are useful in the present invention. Figure 2a is pLJ; Figure 2b is pEm; and Figure 2c is pIp.

Figure 3 is a schematic representation of vectors which express human-LDLR. Each has a different transcriptional element which drives expression of LDLR:

20 LTR-LDLR - viral long term repeat sequences (LTR);  
BA-LDLR - promoter from chicken beta-actin gene (BA);  
H4-LDLR - promoter from human histone H4 gene (H4);  
TK-LDLR - promoter from thymidine kinase gene of herpes simplex virus (TK).

25 Figure 4 is a three-day exposure of a Southern blot in which the effect of extracellular matrix and the time of infection on integration of provirus in rat hepatocyte cultures are shown.

30 Figure 5 represents (panels A-F) cytochemical localization of beta-galactosidase activity in transduced cultures of rat hepatocytes and NIH3T3 cell and (panels

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G-K) liver-specific cytochemical and immunocytochemical stains of rat hepatocyte cultures.

Figure 6 presents results of a Southern analysis of transduced rabbit hepatocytes.

05        Figure 7 presents results of a Northern analysis of transduced rabbit hepatocytes.

Figure 8 is a graphic representation of human parathyroid hormone (PTH) production by transduced rat hepatocytes and of rat albumin production by control  
10        hepatocytes.

#### Detailed Description of the Invention

The present invention is based on development of an effective method of introducing genetic material of interest into hepatocytes and of a method of  
15        transplanting hepatocytes containing the genetic material of interest. Using an appropriate vector, such as a retroviral vector, which includes the genetic material of interest, or other means, it is possible to introduce such genetic material into hepatocytes, where it is  
20        expressed. In particular, it has been demonstrated that DNA of interest can be efficiently and stably introduced into mature cultured hepatocytes, which subsequently express the DNA (produce the encoded polypeptide), and that the transduced hepatocytes can be grafted or  
25        transplanted. In addition, such a vector can be used to introduce genetic material of interest into hepatocytes in vivo, thus avoiding the need to transplant or graft transduced hepatocytes.

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The following is an explanation of isolation of hepatocytes to be transduced ex vivo and grafted or transplanted into a recipient, using the method of the present invention; of vectors useful in introducing  
05 genetic material of interest into hepatocytes, either ex vivo or in vivo; and of the method of the present invention by which hepatocytes are transduced.

#### Isolation of Hepatocytes

In one embodiment of the method of the present  
10 invention, cultured mature hepatocytes are transduced and subsequently grafted or transplanted into a recipient. In this embodiment, hepatocytes are obtained, either from the recipient (i.e., the individual who is to receive the transduced hepatocytes) or from a donor, using known  
15 techniques. In general, this includes removing all or a portion of a liver, from which hepatocytes are removed by in situ perfusion of a collagenase solution. For example, in the case of isolation of hepatocytes from an intact liver, a catheter is inserted into a vein which  
20 either leaves or enters the liver, collagenase solution is perfused through and hepatocytes are released. In the case of a liver biopsy, which results in a cut or exposed surface, a small catheter (or catheters) is inserted into vessels on the open or cut surface. Collagenase solution  
25 is perfused through the catheterized vessels, resulting in release of hepatocytes. Once removed or isolated, the hepatocytes are plated and maintained under conditions (e.g., on appropriate medium, at correct temperature, etc.) suitable for transfection.

30 For example, several methods have been described for isolating highly enriched populations of rat hepatocytes

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and maintaining these cells in culture for extended periods of time. Koch, K.S. and H.L. Leffert, Annals N.Y. Academy of Sciences, 349:111-127 (1980); McGowan, J.A. et al., Journal of Cellular Physiology, 108:353-363 (1981); Bissell, D.M. and P.S. Guzelian, Annals of the New York Academy of Sciences, 349:85-98 (1981); and Enat, R. et al., Proceedings of the National Academy of Sciences, U.S.A., 81:1411-1415 (1984). Such methods can be used to isolate and maintain hepatocytes to be transduced by the method of the present invention. Hepatocytes can also be prepared using a modification of the procedure developed by Barry and Friend, described below and in Example 1, with the perfusion mixture described by Leffert. Leffert, H.L. et al., Methods in Enzymology, 58:536-544 (1979), the teachings of which are incorporated herein by reference.

#### Retroviral Vectors

One limitation of hepatocyte cultures for studying the molecular aspects of processes such as gene regulation has historically been the lack of efficient gene transfer techniques. Conventional methods of transfection are inefficient and toxic to the cells. Tur-Kaspa, R. et al., Molecular and Cellular Biology, 6:716-718 (1986). As described below, recombinant retroviruses have been used to overcome these problems. As a result, it is now possible to efficiently and stably transduce primary cultures of hepatocytes by replication-defective retroviruses. Such replication-defective retroviruses have been used to introduce genetic material of interest into cultured hepatocytes. Transduction is efficient and produces hepatocytes which express the genetic material of interest (i.e., produce the encoded protein or

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polypeptide) and which retain the ability to be transplanted.

Retroviruses are RNA viruses; that is, the viral genome is RNA. This genomic RNA is, however, reverse  
05: transcribed into a DNA intermediate which is integrated very efficiently into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. As shown in Figure 1, the retroviral genome and the proviral DNA have three genes: the gag,  
10: the pol and the env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase); and the env gene encodes viral envelope  
15: glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of virion RNAs.

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral  
20: RNA into particles (the Psi site). Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R., et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proceedings of the National Academy of Sciences, U.S.A., 81:6349-6353  
25: (1984).

If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic RNA. However,  
30: the resulting mutant is still capable of directing the synthesis of all virion proteins. Mulligan and co-

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workers have described retroviral genomes from which these Psi sequences have been deleted, as well as cell lines containing the mutant stably integrated into the chromosome. Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R., et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proceedings of the National Academy of Sciences, U.S.A., 81:6349-6353 (1984). The teachings of these publications are incorporated herein by reference.

The Psi 2 cell line described by Mulligan and co-workers was created by transfecting NIH 3T3 fibroblasts with pMOV-Psi<sup>-</sup>, which is an ecotropic Moloney murine leukemia virus (Mo-MuLV) clone. pMOV-Psi<sup>-</sup> expresses all the viral gene products but lacks the Psi sequence, which is necessary for encapsidation of the viral genome. pMOV-Psi<sup>-</sup> expresses an ecotropic viral envelope glycoprotein which recognizes a receptor present only on mouse (and closely related rodent) cells.

Another cell line is the Psi am line, which are Psi-2-like packaging cell lines. These Psi-am cell lines contain a modified pMOV-Psi-genome, in which the ecotropic envelope glycoprotein has been replaced with envelope sequences derived from the amphotropic virus 4070A. Hartley, J.W. and W.P. Rowe, Journal of Virology, 19:19-25 (1976). As a result, they are useful for production of recombinant virus with a broad mammalian host range, amphotropic host range. The retrovirus used to make the Psi am cell line has an amphotropic host range and can be used to infect human cells. If the recombinant genome has the Psi packaging sequence, the Psi-am cell line is capable of packaging recombinant

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retroviral genomes into infectious retroviral particles. Cone, R. and R. Mulligan, Proceedings of the National Academy of Sciences, USA, 81:6349-6353 (1984).

05 The retroviral genome has been modified by Cone and Mulligan for use as a vector capable of introducing new genes into cells. As shown in Figure 2, the gag, the pol and the env genes have all been removed and a DNA segment encoding the neo gene has been inserted in their place. The neo gene serves as a dominant selectable marker. The  
10 retroviral sequence which remains part of the recombinant genome includes the LTRs, the tRNA binding site and the Psi packaging site. Cepko, C. et al., Cell, 37:1053-1062 (1984).

15 Additional vector constructions which can be used in producing transduced hepatocytes of the present invention are represented in Figure 2 and are described in detail below.

pLJ. The characteristics of this vector have been described in Korman, A.J. et al., Proceedings of the  
20 National Academy of Sciences, USA, 84:2150 (1987). This vector is capable of expressing both the gene of interest and a dominant selectable marker, such as the neo gene. The gene of interest is cloned in direct orientation into a BamHI/SmaI/SalI cloning site just distal to the 5' LTR,  
25 while the Neo gene is placed distal to an internal promoter (from SV40) which is farther 3' than is the cloning site (is located 3' of the cloning site). Transcription from pLJ is initiated at two sites: 1) the  
30 5' LTR, which is responsible for expression of the gene of interest and 2) the internal SV40 promoter, which is responsible for expression of the neo gene. The structure of pLJ is represented in Figure 2a.

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Vector pLJ is represented in Figure 2a. In pLJ, the genetic material of interest is inserted just following the 5' LTR. Expression of this genetic material is transcribed from the LTR and expression of the neo gene is transcribed from an internal SV40 promoter.

pEM. In this simple vector, the entire coding sequence for gag, pol and env of the wild type virus is replaced with the gene of interest, which is the only gene expressed. The components of the pEM vector are described below. The 5' flanking sequence, 5' LTR and 400 bp of contiguous sequence (up to the BamHI site) is from pZIP. The 3' flanking sequence and LTR are also from pZIP; however, the ClaI site 150 bp upstream from the 3' LTR has been ligated with synthetic BamHI linkers and forms the other half of the BamHI cloning site present in the vector. The HindIII/EcoRI fragment of pBR322 forms the plasmid backbone. This vector is derived from sequences cloned from a strain of Moloney Murine Leukemia virus. An analogous vector has been constructed from sequences derived from the myeloproliferative sarcoma virus. The structure of pEM is represented in Figure 2b.

pIp. This vector is capable of expressing a single gene driven from an internal promoter. The structure of pIp is represented in Figure 2c. The construction of these vectors is summarized below. The 5' section of the vector, including the 5' flanking sequences, 5' LTR, and 1400 bp of contiguous sequence (up to the xho site in the gag region) is derived from wild type Moloney Leukemia virus sequence. Shinnick et al., Nature, 293:543 (1918). The difference between the two is that a SacII linker is cloned into an HaeIII restriction site immediately



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adjacent to the ATG of the gag gene. The 3' section of the vector, including the 3' flanking sequences, 3' LTR and 3' contiguous sequence (up to the clal site in the env coding region) is from pZIP. However, there are two  
05 modifications: 1) the clal site has been linked to BamHI and 2) a small sequence in the 3' LTR spanning the enhancer (from PvuII to XbaI) has been deleted. Bridging the 5' and 3' sections of the vector is one of several promoters; each one is contained on a xhoI/BamHI  
10 fragment, and each is capable of high level constitutive expression in most tissues. These promoters include beta-actin from chicken (Choudory, P.V. et al., CSH Symposia Quantitative Biology, L.I. 1047 (1986), and thymidine kinase from Herpes Simplex Virus, histone H4  
15 from human (Hanly, S.M. et al., Molecular and Cellular Biology, 5:380 (1985)). The vector backbone is the HindIII/EcoRI fragment from pBR322. The gene of interest is cloned into the BamHI site in direct orientation, just downstream from the internal promoter.

20 Vectors without a selectable marker can also be used to transduce endothelial cells with genetic material of interest. Such vectors are basically simplifications of the vectors previously described, in which there is such a marker. Vector pEM is represented in Figure 2b; as  
25 represented, the main components of the vector are the 5' and 3' LTR, and the genetic material of interest, inserted between the two LTRs.

#### Retroviral vectors useful for ex vivo modification of hepatocytes

30 Four additional recombinant retroviruses suitable for introducing genetic material of interest into

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cultured hepatocytes and expressing biologically significant amounts of the encoded protein or polypeptide are also represented in Figure 3, with specific reference to the human LDLR gene. These vectors, which included  
05 the human LDLR gene, have been used to efficiently transduce hepatocytes, which then expressed levels of LDLR equal to normal endogenous levels. These vectors will be illustrated with reference to the LDLR gene, but any nucleotide sequence of interest can be incorporated  
10 into the retroviruses and introduced into hepatocytes.

As shown, each vector differs in the transcriptional elements used to drive expression of the gene. These are described in detail in Example IV. Briefly, in the vector LTR-LDLR, transcription should begin at the 5'  
15 LTR, with the result that a single full length viral transcript, in this case, one which expresses LDLR, is produced. In the remaining vectors, expression of LDLR should be driven by transcripts initiated from transcriptional control sequences located internal to the  
20 proviral transcriptional unit.

Each of these latter vectors differs in the transcriptional elements responsible for transcription: BA-LDLR contains the promoter from chicken beta-actin gene; H4-LDLR contains the promoter from the human  
25 histone H4 gene; and TK-LDLR contains the promoter from the thymidine kinase gene from herpes simplex virus. Each of the three vectors also contains a deletion of viral transcriptional enhancer sequences located in the 3' LTR, in order to reduce the amount of viral  
30 transcription which occurs after reverse transcription and integration of the recombinant provirus. Human LDLR

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coding sequences for all four vectors were derived from a full length human LDLR cDNA insert.

As described in Example IV, use of these vectors in transducing hepatocytes resulted in levels of viral-directed LDLR RNA in the transduced cells that exceeded endogenous levels (50-100X). The amount of functional receptor produced, however, was less than or equal to normal endogenous levels for most vectors, possibly due to insufficient RNA processing or transport or diminished translational efficiency.

Because a potential problem with the ex vivo method of hepatocyte modification is the fact that only a fraction of the total hepatocytes isolated can be engrafted, additional retroviral vectors that express higher levels of the encoded protein (here, LDLR) may be useful. Such vectors may be useful, for example, as a means of decreasing the number of transplanted cells required for production of useful quantities of the encoded protein. For example, each vector can include specialized transcriptional control sequences which are internal to the proviral transcriptional unit. This makes it possible to provide variation in the level of transcription of the nucleotide sequence of interest. Of particular interest here are transcriptional elements from genes expressed at high levels in liver cells (e.g., alpha-fetoprotein, albumin).

To enhance the translational efficiency of the chimeric nucleotide sequence of interest RNAs generated by the vectors, (e.g., by generating more translatable RNAs), selected 5' and 3' nontranslated sequences from a well-characterized gene (e.g., RU-5 region of a human retrovirus) or the authentic 5' and 3' nontranslated

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sequences of the nucleotide sequence of interest can also be included in the retroviral vectors used.

Because it is possible that constitutive production of very high levels of the encoded protein (e.g., LDLR) may result in toxicity to cells, it may also be appropriate to include in the vector sequences from the nucleotide sequence of interest (or gene) which confer transcriptionally-mediated end-product repression. For example, in the case of the LDLR gene, sequences (called the steroid responsive elements) which confer transcriptionally-mediated, end-product repression by sterols can be included in the vector.

Retroviral vectors for in vivo modification of hepatocytes

It is also possible to use vectors, particularly recombinant retroviral vectors, to transduce hepatocyte cells in vivo. For example, one strategy for targeting the LDLR gene to hepatocytes can be based on the presence of the asialoglycoprotein (ASGP) receptor on hepatocytes. This receptor, which is specifically expressed in hepatocytes, is involved in the uptake and catabolism of glycoproteins that have had their terminal sialic acids removed, thereby exposing penultimate galactose residues. Glycoprotein-receptor complexes are internalized by receptor mediated endocytosis. Asialoglycopeptide-protein conjugates and asialoglycopeptide-coated vesicles have been used to specifically deliver a variety of bioactive agents to the liver in vivo. Aitię, A.D. et al., Proceedings of the National Academy of Sciences, USA, 27:5923-5927 (1980); Fiumw, L. et al., FEBS Letter,

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103:47-51 (1979); Hildenbrandt, G.R. and N.N. Aronson, BBA, 631:499-502 (1980).

For example, one approach to targeting delivery of genes to hepatocytes can be based on the modification of existing retroviruses to make them ligands for the ASGP receptors. The envelope proteins from murine leukemia viruses are complex glycoproteins that have a high content of sialic acid. Internalization of virus occurs through the specific interaction of the viral envelope with a cell surface receptor, followed by receptor-mediated endocytosis of the virus/receptor complex.

Hepatocyte-specific transduction may be possible if modified virions that are specifically internalized by the ASGP receptor are developed and used. For example, it is possible to use viruses whose envelope protein is devoid of sialic acid, thereby rendering them ligands for the ASGP receptor. One approach is to enzymatically remove the terminal sialic acids from intact virions with neuraminidase. Alternatively, it is possible to construct viral producer lines with genetically modified envelope genes that code for glycoproteins with terminal galactose residues. It is possible to construct chimeric envelope genes that encode fusion proteins in which the carboxy terminal sequences are derived from the 3' portion of the envelope gene and the amino terminal sequences are derived from genes of known ligands for ASGP receptor. In addition, it is possible to use lectin-resistance selection systems to isolate mutants of the viral producer lines that are incapable of adding terminal sialic acids to N-linked chains. . Viruses produced from these lines should bind to the ASGP receptor. Transduction of cells that express ASGP

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receptor with these modified virions can be tested in vitro and in vivo.

Another approach to the targeted delivery of genes to hepatocytes has recently been described by Wu. Wu, G.Y. and C.H. Wu, Journal of Biological Chemistry, 262:4429-4432 (1987). Wu coupled pSV2CAT plasmid to a ligand of the ASGP receptor called asialoorosomucoid (ASOR) and demonstrated in HEP C2 cells uptake of the conjugates via the ASGP receptor and expression of CAT activity. After administration of this conjugate to rats, specific, but transient, expression of CAT was demonstrated in liver homogenates. It is possible that this method can be modified to produce a method by which recombinant retroviral vectors or episomal vectors can be introduced into hepatocytes in vivo. The efficiency and specificity of this delivery system can be assessed, for example, by transferring an expression vector that stably expresses a product which can be detected in situ by direct analysis of the product (e.g., beta-galactosidase).

Successful application of in vivo targeting requires stable expression of the transferred gene. Transduction of hepatocytes in vivo with retroviruses that have been modified to allow for uptake via the ASGP receptor will require integration of the provirus into genomic DNA. Integration is rare in the quiescent liver, however. The efficiency of retroviral integration may be improved by exposing the recipient to virus following partial hepatectomy. During this time, the residual hepatocytes undergo rapid proliferation. This, however, may not be practical clinically. One way to avoid this problem is to modify the retroviral vector in such a way as to

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promote its persistence as an episome in the form of a double stranded DNA circle. This can be done by incorporating the cis (oriP) and trans (EBNA) sequences of Epstein Barr Virus that are required for plasmid replication into a retrovirus vector. It is also possible to use other sequences (called autonomously replicating sequences) isolated from the eukaryotic (e.g., mouse) genome which have been shown to drive the autonomous replication of plasmids. In these cases, vectors containing deletions of sequences within the inverted repeats of the long terminal repeats which prevent proviral integration into the host chromosome, will be used.

#### Introduction of Genetic Material into Hepatocytes

Genetic material of interest has been incorporated into cultured hepatocytes and expressed in the resulting genetically engineered hepatocytes, as described below and in the examples.

Genetic material which can be incorporated into hepatocytes according to the method described can be:

- 1) genetic material (DNA or RNA) which is present in and expressed at biologically effective levels (levels sufficient to produce the normal physiological effects of the polypeptide it encodes) in normal hepatocytes, but present in or expressed in less than normal quantities in the hepatocytes prior to stable transfer of genetic material of interest into them by the method of the present invention;
- 2) genetic material not present in normal hepatocytes; or
- 3) genetic material present in normal hepatocytes but not expressed at biologically

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effective levels in such cells, alone or in any combination thereof.

The genetic material incorporated into and expressed by hepatocytes can also, optionally, include genetic material encoding a selectable marker, thus making it possible to identify and select cells which contain and express the genetic material of interest.

Thus, DNA or RNA introduced into cultured hepatocytes of the present invention includes the genetic material (DNA or RNA) of interest and, optionally, genetic material encoding a selectable marker; such DNA or RNA is referred to as incorporated genetic material (or incorporated DNA, incorporated RNA). Hepatocytes containing incorporated genetic material are referred to as transduced hepatocytes; they express the DNA or RNA of interest and produce the encoded protein or polypeptide.

Exogenous DNA encoding a polypeptide or protein of interest and, optionally, a selectable marker (e.g., neo, which encodes neomycin resistance) is incorporated in vitro into hepatocytes as described below and in Examples I-III. Hepatocytes isolated as described previously are plated at subconfluent density on matrix substrata and maintained in hormonally defined media, such as that described by Enat et al., the teachings of which are incorporated herein by reference. Enat, R., et al., Proceedings of the National Academy of Sciences, USA, 81:1411-1415 (1984). The media is changed as needed to maintain the hepatocytes.

Cells are subsequently infected with an amphotropic retrovirus which contains DNA of interest (e.g., DNA encoding a polypeptide whose expression in hepatocytes is desired) and, optionally, DNA encoding a selectable



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marker to be incorporated into the hepatocytes. The hepatocytes are infected with the recombinant retrovirus (and thus transduced with the DNA of interest) by exposing them to virus which has a recombinant genome. This results in infection of the cells by the recombinant retrovirus. It is possible to optimize the conditions for infection of the hepatocytes by using a high titer amphotropic virus.

A cell line which produces recombinant amphotropic retrovirus having a recombinant genome is used to infect hepatocytes. The recombinant genome can include a variety of components, but in general is comprised of two LTRs and, in place of the gag, the pol and the env sequences, a second promoter sequence; in some cases, it also includes a gene encoding a selectable marker (e.g., neo).

Viral stocks, to be used in introducing genetic material of interest into hepatocytes, are harvested, as described above, supplemented with Polybrene (Aldrich) and added to the culture of hepatocytes. If the titer of the virus is high (e.g., approximately  $10^6$  cfu per ml.), then virtually all hepatocytes will be infected and no selection of transduced hepatocytes is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. If a selectable marker is used, after exposure to the virus, the cells are grown to confluence and split into selective media (e.g., media containing G418 if the selectable marker is neo, media containing histidinol and no histidine if the selectable marker is his).

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The neo gene is a bacterial gene derived from the transposon Tn5, which encodes neomycin resistance in bacteria and resistance to the antibiotic G418 in mammalian cells. This neo gene acts as a dominant selectable marker; its presence in a mammalian cell converts the cell into one which will grow in the presence of G418. (If it is not present, the cell dies in the presence of G418.) As a result, the presence of this gene in a mammalian cell can be determined by culturing cells in media which contains G418.

The recombinant retroviral vectors having the neo gene also have a cloning site. As a result, genetic material of interest can be introduced into the vector, incorporated into hepatocytes and expressed by hepatocytes transduced with the recombinant retrovirus (referred to as hepatocytes containing incorporated genetic material). At the BamHI cloning site, it is possible to insert genetic material of interest. As described above, hepatocytes have been transduced with the gene encoding beta-galactosidase from E. coli. The efficiency of transduction was assessed, as described in Example III. Expression of the beta-galactosidase gene was also assessed and is detailed in Example III.

For example, a helper-free amphotropic producer is grown in tissue culture to a confluent density in Dulbecco's Modified Eagle's Medium (DME) supplemented with 10% calf serum (CS). Fresh media is added and subsequently the media is harvested. The spent media (or viral stock) is filtered to remove detached producer cells and is used immediately to infect cells or is stored (e.g., at -70°C) for later use.

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Media is removed from a subconfluent plate of hepatocytes (recipient hepatocytes) and quickly replaced with viral stock which contains Polybrene (Aldrich). Subsequently, this is removed and replaced with fresh media. Thus, the media used is a viral supernatant and the recombinant genome of the infectious virus includes DNA of interest. The infection procedure results in hepatocytes which express the DNA encoding a gene product of interest and, optionally, a selectable marker.

In one embodiment, hepatocytes are exposed to media containing infectious virus produced in Psi am cells; the infectious virus contain a recombinant genome having the genetic material of interest. The recombinant genome in one instance includes genetic material encoding a protein or a polypeptide (e.g., the receptor for low density lipoproteins; ornithine transcarbalyase) and, optionally, a gene encoding a dominant selectable marker (e.g., the neo gene which encodes neomycin resistance). As a result, the hepatocytes are transduced -- that is, the genetic material of interest (for example, DNA encoding a polypeptide or a protein of interest) and, optionally, the neo gene are stably introduced into the hepatocytes. The transduced hepatocytes express the encoded protein or polypeptide and, if the neo gene is present, express it, resulting in cells having the selectable trait.

One embodiment of the present invention is described in detail in the Examples. Briefly, hepatocytes were prepared using a modification of the method of Berry and Friend with the perfusion mixture of Leffert. Berry, M.N. et al., J. Cell. Biol., 43:506-520 (1969); Leffert, H.L. et al., Methods Enzymol., 58:536-644 (1979). The resulting hepatocytes were plated at a density of  $3-4 \times 10^4$

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cells/cm<sup>2</sup> onto Primaria plates substratum in hormonally defined media supplemented with 10% fetal bovine serum. The media was replaced with fresh hormonally defined media, which was subsequently changed periodically.

05 Spent media was obtained by harvesting DME media supplemented with 10% calf serum in which a helper-free amphotropic producer of the BAG virus was cultured. Conditions for infection of the hepatocytes were optimized through the use of BAG, which is a high titer

10 amphotropic virus encoding E. coli beta galactosidase. The producer coexpressed beta-galactosidase from E. coli and the bacterial neo gene. The spent media was filtered to remove detached producer cells and used as viral stock to infect hepatocytes.

15 Cells were infected by removing media from a sub-confluent plate of hepatocytes (recipient hepatocytes) and replacing it with viral stock. Hepatocyte cultures were infected in this way for approximately 12 hours. Hepatocytes containing the DNA of interest (i.e., DNA

20 encoding beta-galactosidase) and the neo gene were isolated by culturing on media containing the antibiotic G418. Those into which the recombinant retrovirus was effectively introduced by infection, referred to as transduced hepatocytes, produce beta-galactosidase and

25 are neomycin resistant. The ability of hepatocytes transduced with the recombinant retrovirus having the beta-galactosidase gene to produce beta-galactosidase has been assessed in vitro. This assesement is described in Example III.

30 Rat hepatocytes have also been transduced with the gene encoding human parathyroid hormone (hPTH) and shown

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to express the encoded hormone. This is described in Example I and results are presented in Figure 8.

As a result, it has been demonstrated that transduced hepatocytes secrete a polypeptide (beta-galactosidase) which is normally not secreted by hepatocytes. A similar approach can be used to introduce any genetic material into hepatocytes in such a manner that the encoded product is made and to assess expression of the incorporated genetic material.

For example, hepatocytes have been transduced as a result of exposure to media containing infectious virus in which the recombinant genome includes DNA encoding human LDLR. This is described in detail in Example IV. Hepatocytes were infected with four LDLR virus preparations (each including one of the vectors represented in Figure 3) and subsequently analyzed for gene transfer and LDLR expression.

The same vectors can be used in the method of the present invention, to introduce into cultured hepatocytes any nucleotide sequence or gene of interest. Alternatively, other vectors can be used, as can other means of introducing genetic material of interest into cultured hepatocytes.

As explained previously, it may also be possible to introduce genetic material of interest into hepatocytes in vivo. As described, a recombinant retrovirus in which the genome includes the genetic material of interest would be targeted to hepatocytes, with the result that after introduction into an individual's body (e.g., by intravenous injection), the retrovirus is specifically taken up by hepatocytes. Once taken up by hepatocytes, the recombinant retroviral genome will be expressed,

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resulting in production of the protein or polypeptide encoded by the genetic material of interest. Preferably, the virus used to introduce the genetic material of interest is modified (e.g., through inclusion of sequences, referred to as autonomously replicating sequences or ARS) in such a manner that integration of the provirus into the host chromosomes does not occur. As a result, replication will occur episomally.

10 Use of Dominant Selectable Markers in the Introduction of Genetic Material Encoding Polypeptides

In addition to genetic material of interest, a vector can include genetic material encoding a selectable marker, the presence of which makes it possible to identify and select for cells transduced with the genetic material of interest. As described previously and in Example III, the neo gene, which is such a marker, has been used for this purpose. It is also possible to use dominant selectable markers other than the neo gene to introduce genetic material into hepatocytes. For example, the His D gene can be used for this purpose. The His D gene is a bacterial gene from Salmonella and encodes histidinol dehydrogenase, a polypeptide which converts histidinol to histidine. Histidine is an essential amino acid; histidinol is an alcohol analogue of histidine and can be converted to histidine under the proper metabolic conditions. If cells are grown in media containing histidinol but lacking histidine, those cells having the His D gene can convert histidinol to histidine. Because histidine is essential to their function, those cells which have the His D gene (and thus

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can make histidine) will survive and those lacking the gene will not.

A retrovirus vector having the His D gene has been used to infect keratinocytes. The keratinocytes containing His D gene were selected by growing these cells in media lacking histidine but containing histidinol. As expected, keratinocytes having the His D gene formed colonies and grew to confluence; those lacking the gene did not. In fact, such cells occurred at a much higher frequency than those in which the neo gene was included. These same techniques are useful in selecting fibroblasts containing DNA of interest.

As a result of this work, it is also possible to use independent dominant selectable markers (e.g., the neo gene and the His D gene) to introduce more than one type of new genetic material into hepatocytes. In the case of gene products which have two different subunits, for example, separate dominant selectable markers can be used to introduce the genetic information encoding the two subunits. In addition, two or more dominant selectable markers can be used in the case of gene products which need to be specifically cleaved or processed in order to become active. A gene encoding the necessary processing enzyme can be introduced along with the gene encoding the polypeptide requiring such processing. This would enable hepatocytes to process the polypeptide hormone.

#### Other Vehicles for the Introduction of Genetic Material of Interest into Hepatocytes

It is also possible to use vehicles other than retroviruses to genetically engineer or modify hepatocytes. Genetic information of interest can be

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introduced by means of any virus which can express the new genetic material in such cells. For example, SV40, herpes virus, adenovirus and human papilloma virus can be used for this purpose. DNA viruses can also be used to  
05 introduce genetic material of interest, as well as a gene encoding a selectable marker, into hepatocytes according to the method of the present invention.

#### Transplantation of Transduced Hepatocytes

Hepatocytes expressing the incorporated genetic  
10 material are grown to confluence in tissue culture vessels; removed from the culture vessel; and introduced into the body. This can be done surgically, for example. In this case, the tissue which is made up of transduced hepatocytes capable of expressing the nucleotide sequence  
15 of interest is grafted or transplanted into the body. For example, it can be placed in the abdominal cavity in contact with/grafted onto the liver or in close proximity to the liver. Alternatively, the transduced hepatocyte-containing tissue can be attached to microcarrier beads,  
20 which are introduced (e.g., by injection) into the peritoneal space of the recipient. This approach has been shown to be successful by transplantation of wild type hepatocytes into a strain of rats (Nagase analbuminemic rats) which are deficient in albumin  
25 synthesis and demonstration of moderate levels of albumin in serum of transplanted animals. Direct injection of genetically modified hepatocytes into the liver may also be possible.

Once introduced into the body of an individual, the  
30 transduced hepatocytes provide a continuous supply of the hormone, enzyme or drug encoded by the genetic material



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of interest. The amount of the hormone, enzyme or drug supplied in this way can be modified or regulated as needed (e.g., by using external cues or factors which control or affect production, by controlling the size of the graft or the quantity of fibroblasts introduced into the body, or by removing the graft).

Genetically modified hepatocytes have been implanted into WHHL rabbits which lack LDL receptor function. Hepatocytes were isolated and infected with the LTR-LDLR recombinant retrovirus. The transfected hepatocytes were harvested and injected into the portal vein of WHHL rabbits. Results demonstrated that serum cholesterol levels decreased 30% over a 3-day period, suggesting that it is possible to at least partially correct the metabolic abnormalities in the WHHL rabbit by transplantation of genetically modified hepatocytes.

#### Uses of Genetically Modified Hepatocytes Having Incorporated Genetic Material

The present invention makes it possible to genetically engineer hepatocytes in such a manner that they produce a gene product (e.g., a polypeptide or a protein) of interest in biologically significant amounts. The hepatocytes formed in this way can serve as a continuous drug delivery system to replace present regimens, which require periodic administration (by ingestion, injection, etc.) of the needed substance.

Incorporation of genetic material of interest into hepatocytes would be particularly valuable in the treatment of inherited disease and the treatment of acquired disease. In the case of inherited diseases, this approach is used to provide genetically modified

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hepatocytes which contain DNA encoding a protein or polypeptide which an individual's hepatocytes are unable to make correctly. For example, this could be used in treating urea cycle disorders. Hepatocytes of the present invention can also be used in the treatment of genetic diseases in which a product (e.g., LDL receptor) normally produced by the liver is not produced or is made in insufficient quantities. Here, hepatocytes transduced with a DNA encoding the missing or inadequately produced substance can be used to produce it in sufficient quantities. In this case, the transduced hepatocytes would produce LDL receptor and thus provide a means of preventing or treating familial hypercholesterolemia, which is an inherited disease in which the primary genetic defect is an abnormality in the expression or function of the receptor for low density lipoproteins. This leads to elevated levels of serum cholesterol and the premature development of coronary artery disease. The transduced hepatocytes could be used to produce sufficient quantities of the LDL receptor to overcome the underlying defect.

There are also acquired diseases for which treatment can be provided through use of genetically engineered hepatocytes. For example, such cells can be used in treating the coagulopathy associated with liver failure. In this case, hepatocytes having incorporated in them a gene encoding one or more clotting factors would correct the acquired deficiency of these factors which leads to bleeding. It may also be possible to treat viral hepatitis, particularly hepatitis B or nonA-nonB hepatitis, by gene transfer. For example, using the method of the present invention, a gene encoding an

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anti-sense gene could be introduced into hepatocytes to inhibit viral replication. In this case, a vector including a structural hepatitis gene in the reverse or opposite orientation would be introduced into  
05 hepatocytes, resulting in production in transduced hepatocytes of an anti-sense gene having the correct orientation.

The present invention will now be illustrated by the following examples, which are not to be seen as limiting  
10 in any way.

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EXAMPLE I Hepatocyte Isolation and Culture

Rat hepatocytes were prepared by the procedure of Berry and Friend, using the perfusion mixture of Leffert. Berry, M. N. and D. S. Friend, Journal of Cell Biology, 43:506-520 (1969); Leffert, H. L. et al., Methods in Enzymology, 58:536-544 (1979). Male Sprague-Dawley rats weighing between 200 and 250 gms were used as the source of hepatocytes. Cells were plated at a density of  $4 \times 10^4$  cells/cm<sup>2</sup> onto one of several matrix substrata in hormonally defined media supplemented with 10% fetal bovine serum. Enat, R. et al., Proceedings of the National Academy of Sciences, USA, 81:1411-1415 (1984). Four hours later the media was replaced with fresh hormonally defined media which was subsequently changed every 24 hours during the duration of the experiment. The following substrata were used: 1) Tissue culture plastic - Primaria plates from Falcon Co. were used without additional preparation; 2) Type I collagen - 10 cm tissue culture dishes were coated with type I collagen prepared from rat tail tendons. Michalopoulos, G. and H. Pitot, Exp. Cellular Research, 94:70-78 (1975). Briefly, collagen was solubilized in 0.1% acetic acid (3 mg/ml) and applied to plates (1 ml/10 cm plate) which were exposed to NH<sub>3</sub> vapors, air dried, sterilized by gamma irradiation (10,000 rads), and hydrated with media; 3) Laminin - Purified laminin from Collaborative Research Inc. (Waltham, MA) was applied to tissue culture plates according to the recommendations of the manufacturer; 4) Type IV collagen - 10 cm dishes coated with purified type IV collagen were kindly provided by Dr. L.M. Reid (Albert Einstein College of Medicine).

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EXAMPLE II Virus Preparation and Hepatocyte Infection

A helper-free amphotropic producer of the BAG virus was provided by Dr. C. Cepko (Harvard). The retroviral vector used to make this producer has been described by  
05 Cepko and co-workers. Price, J., Proceedings of the National Academy of Sciences, U.S.A., 84:156-160 (1987). It coexpressed beta-galactosidase from E. coli and the bacterial gene that confers resistance to neomycin in prokaryotes and to G418 in eukaryotes (neo). The  
10 producer was maintained in Dulbecco's modified Eagle's Medium supplemented with 10% calf serum. Unconcentrated viral stocks were prepared and titered as described by Mann. Mann, R. et al., Cell, 33:153-159 (1983). Titers ranged from  $1-4 \times 10^5$  cfu/ml. Hepatocyte cultures were  
15 infected for 12 hours with viral stocks (5 ml of viral stock/10 cm plate of hepatocytes) containing 8 ug/ml of Polybrene (Aldrich). Transduction efficiency was optimized with respect to the time of exposure to virus and the matrix substrate on which the hepatocytes were  
20 plated.

EXAMPLE III Assessment of Efficiency of Transduction

The efficiency of transduction was initially assessed by directly measuring the integration of provirus.

25 Southern Analysis

High-molecular-weight cellular DNA from transduced cultures of hepatocytes was isolated as described previously. Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold  
30 Spring Harbor, N.Y. (1982). Aliquots (7.5 ug) were digested with the restriction endonuclease Kpn I. Kpn I

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has recognition sites in the proviral long terminal repeats; consequently, each integrated provirus will be contained in a 6.9 Kb restriction fragment, irrespective of the site of integration.

05       The restriction fragments were resolved by electrophoresis in 1% agarose gels and analyzed according to the method of Southern using standard procedures and a probe that is complementary to sequences unique to the provirus (i.e., the neo gene). Maniatis, T. et al., Molecular  
10 Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1982). The blot was probed with the Bam/Hind III fragment of the neomycin gene that was labelled to high specific activity with <sup>32</sup>P-dCTP using the random primer method. Feinberg, A.P.  
15 and B. Vogelstein, Anal. of Biochemistry, 132:6-13 (1983). The intensity of the resulting band on the autoradiograph is proportional to the number of proviral integrants in the population.

Figure 4 shows the effect of extracellular matrix  
20 used and of the time of infection on integration of provirus in hepatocyte cultures. Panel A presents a Southern blot of hepatocytes isolated from a single collagenase perfusion which were cultured on 10 cm.  
plates coated with one of several forms of matrix  
25 substrata (type I collagen, laminin, type IV collagen, and tissue culture plastic) and infected on days 1, 2, 3, 4, or 5. Infection was carried out using fresh preparations of viral stocks and plates were analyzed for copy number of integrated provirus 48 hours after the infection was initiated.  
30

A three day exposure of a Southern blot is shown. A single band was visualized in each lane; the area of the

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autoradiograph containing this band is shown. Lanes 1-5 indicate the days that the cells were infected. The top four series of bands represent hepatocytes cultured on different forms of matrix: Col I - type I collagen, 05 Lam-laminin, Col IV - type IV collagen, and TCP-tissue culture plastic. The bottom series of bands shows an identical analysis of NIH3T3 cells infected with the same viral stocks used to infect the hepatocyte cultures.

Hepatocytes on each matrix substrata exhibited a 10 consistent pattern of susceptibility to transduction; proviral integration increased from virtually undetectable on day 1 to maximal on days 2 or 3, and subsequently diminished to low levels by day 5. Maximal proviral integration, which was essentially independent of matrix, 15 occurred when cultures were infected on day 2 for cells on tissue culture plastic, or day 3 for cells on type I collagen, laminin, or type IV collagen.

NIH3T3 cells were infected with the same viral stocks used to infect hepatocytes (Fig. 1, panel A). 20 Southern analysis demonstrated little variation in the titer of the viral stocks; the estimated proviral copy number ranged from 0.5 to 0.7 copies/cell. This estimate of copy number was based on a comparison with samples with known quantities of standard plasmid (Fig. 4, panel 25 B) and the assumption that NIH3T3 cells are hypotetraploid.

The samples with known quantities of standard plasmid were made by mixing varying amounts (2 pg and 10 pg) of the purified BAG plasmid with 7.5 ug of uninfected 30 NIH3T3 DNA. Analysis showed a single band, which comigrated with the bands shown in panel A. The data from panels A and B were derived from a three day

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exposure of the same Southern blot. It was estimated that 2 pg and 10 pg of plasmid in 7.5 ug of NIH3T3 DNA correlates to approximately 0.3 and 1.2 copies of provirus/cell, respectively. The estimated copy number of proviral integrants in maximally infected hepatocytes (e.g., Figure 4, Lam, lane 3) is approximately 0.2 copies/cell, assuming that the DNA content of NIH3T3 cells is equal to that of hepatocytes. This assumption is probably valid since the majority of hepatocytes in culture are either tetraploid or octaploid. Tomita, Y., et al., Exp. Cell Res., 135:363-370 (1981).

#### Cytochemical and Immunocytochemical Procedures

A series of liver-specific cytochemical and immunochemical stains was used to document the cellular composition of the hepatocyte cultures. All analyses were performed on three day old cultures of hepatocytes plated on type I collagen.

Cells infected with the BAG virus constitutively produce high levels of cytoplasmic beta-galactosidase. Price, J. et al., Proceedings of the National Academy of Sciences, U.S.A., 84:156-160 (1987). Activity of beta-galactosidase was detected in situ with the substrate 5-bromo-4-chloro-3-indolyl-D-galactosidase, which forms a blue precipitate in infected cells. Price, J. et al., Proceedings of the National Academy of Sciences, U.S.A., 84:156-160 (1987).

Duplicate cultures of infected hepatocytes were analyzed in situ for retrovirus-transduction (and expression) by the cytochemical stain for beta-galactosidase. Price, J. et al., Proceedings of the National Academy of Sciences, U.S.A., 84:156-160 (1987). This procedure



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specifically labels cells that express viral directed beta-galactosidase; endogenous beta-galactosidase is not detected. Figure 5 (panels A-E) shows cytochemical localization of beta-galactosidase activity in transduced  
05 cultures of hepatocytes and NIH3T3 cells. Panels A-E show hepatocyte cultures plated on type I collagen and infected on days 1, 2, 3, 4, or 5 (panels a-e, respectively). Panel f shows a population of NIH3T3 cells which had been infected with the same viral stocks used  
10 to infect hepatocytes on day 3.

Results showed that labeled cells are often found in groups of 2, probably representing infection and integration into a dividing cell with labeling of the two daughter cells. In addition, expression of beta-  
15 galactosidase, as determined by the intensity of staining, is quite variable, but tends to be consistent within members of a pair of labeled cells (e.g., see panel C). The efficiency of transduction, as measured cytochemically, exhibited the same dependence on time in culture  
20 as was demonstrated by Southern analysis. The fraction of labeled cells increased from less than 1% in cultures infected on day 1 to approximately 25% when infected on day 3; the transduction efficiency dropped dramatically in cultures infected on the next 2 days (days 4 and 5).

25 Similar analysis of NIH3T3 cells infected with the same viral stock used to infect day 3 hepatocytes indicated that approximately 50% of the cells were labelled. This is consistent with the estimated efficiency of transduction based on Southern blot analysis (Figure 4,  
30 panel A).

Immunocytochemical localization of UDP-glucuronosyl-transferase and asialoglycoprotein receptor was performed

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in the culture dishes using horseradish peroxidase conjugated to protein A (from *Staphylococcus aureus*) and diaminobenzidine cytochemistry at pH 7.4 to detect peroxidase activity. Novikoff, P.M. et al., Journal of Cell Biology, 97:1559-1565 (1983). Monospecific IgG to rat UDP-glucuronosyltransferase was purified from rabbit antiserum. Chowdhury et al. used immunocytochemical techniques to determine the distribution of UDP-glucuronosyltransferase in the liver. This membrane-bound enzyme is present exclusively in hepatocytes and is localized to the endoplasmic reticulum and nuclear membrane. Chowdhury, J.R. et al., Proceedings of the National Academy of Sciences, U.S.A., 82:2990-2994 (1985). Dr. R. Stockert (Albert Einstein College of Medicine) kindly provided monospecific antibody to rat asialoglycoprotein receptor. Controls for the immunocytochemical experiments included exposure of cells to pre-immune rabbit antisera, followed by identical procedures as those employed for specific rabbit antibody. Immunocytochemical analysis of cultured hepatocytes using the monospecific polyclonal antibody to UDP-glucuronosyltransferase shows reaction product distributed in cytoplasmic clumps and at the periphery of the nucleus in greater than 95% of the cells (Figure 5, panel G); these reactive sites correspond to the endoplasmic reticulum and the nuclear envelope, respectively. No reaction product is seen in experiments performed with preimmune rabbit IgG (Figure 5, panel i).

#### Asialoglycoprotein receptor

This well described receptor is specifically expressed in hepatocytes. Immunocytochemical analysis in

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rat liver localizes this receptor to a domain of the plasma membrane which borders the sinusoids; under light microscopy the receptor is seen at the perimeter of the hepatocyte along its sinusoidal face. Genze, J.E. et al., Journal of Cellular Biology, 92:867-870 (1982);  
05 Matsuura, S. et al., Journal of Cellular Biology, 95:864-875 (1982). The level of asialoglycoprotein receptor decreases in culture; however, it is still demonstrated in virtually all cells of a three-day-old  
10 hepatocyte culture. Reaction product is seen as a dense line in focal regions of the hepatocyte periphery (Figure 5, panel H, in which results of localization with a monospecific rabbit antibody are shown). This characteristic staining is absent in experiments with control (pre-  
15 immune) rabbit serum (Figure 5, panel i).

#### Glucose-6-phosphatase

This glycolytic enzyme is a well recognized cytochemical marker for hepatocytes. It can be detected in virtually all hepatocytes of liver sections. However,  
20 there is marked regional variation in enzyme activity; the greatest activity is found in the periportal region. Sasse, D., "Regulation of Hepatic Metabolism and Intra- and Intercellular Compartmentalization, eds. Thurman, R.G., Kauffman, F. C. and Jungermann, K. (Plenum Press,  
25 NYC), pp. 57-86 (1986).

Glucose-6-phosphatase activity was detected in three-day-old hepatocyte cultures by the lead phosphate enzyme cytochemical procedure. Wachstein, M. and E. Meisel, J. Histochem. Cytochem., 4:592 (1956).

30 Characteristic brown/black cytoplasmic staining was seen in greater than 95% of the cells (Figure 5, panel

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J). As expected, there was marked cell-to-cell variation in enzyme activity. Activity was not detected in pure cultures of nonparenchymal cells such as fibroblast.

#### Peroxisomes

05       The method of Novikoff et al. was used to visualize the distribution of peroxisomes in hepatocyte cultures. Novikoff, A. B. et al., J. Histochemistry and Cytochemistry, 20:1006-1023 (1972). These small cytoplasmic structures (approximately 0.5 microns in diameter) are  
10       found specifically in hepatocytes (in the context of the liver) and are visualized by cytochemical staining for catalase. DeDuve, C. et al., Physiol. Rev., 46:323-357 (1966).

15       Greater than 95% of the cells in the culture tested demonstrated numerous catalase-positive peroxisomes, which appeared as dot-like structures distributed randomly throughout the cytoplasm (Figure 5, panel K). Peroxisomes were not detected when pure cultures of nonparenchymal cells (e.g., fibroblasts) were analyzed.

20       EXAMPLE IV       Retrovirus-Mediated Gene Transfer to  
                          Correct LDLR Deficiency  
              Isolation, Maintenance and Infection of Hepatocytes  
              Newborn New Zealand white (NZW) rabbits and  
25       Watanable heritable hyperlipidemic (WHHL) rabbits (3-5 days old weighing 50-80 gms) were used as the source of  
30       hepatocytes. The WHHL rabbit has been shown to be deficient in functional LDLR activity due to an in-frame deletion of a portion of the LDLR structural gene. Yamamoto, T. et al., Science, 232: 1230-1237 (1986). NZW rabbits have been used as controls in most previous

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studies of the WHHL rabbit. Newborn WHHL rabbits were derived from matings between homozygous deficient males and females and were kindly provided by Dr. Knapka (NIH). Four WHHL rabbits from 2 litters (2 rabbits/litter) were used in these studies (named WHHL 1-4). Newborn NZW rabbits were purchased from Pine Acres Rabbitry (West Brattleboro, Vermont). Hepatocytes were prepared using a modification of the procedure of Berry and Friend with the perfusion mixture of Leffert. Berry, M.N. and D.S. Friend, Journal of Cell Biology, 43:506-520 (1969). Leffert, H.L. et al., Methods in Enzymology, 58:536-544 (1979). Collagenase perfusions were performed retrograde as described by Clayton and Darnell for the preparation of adult mouse hepatocytes. Clayton, D.F. and J.E. Darnell, Jr., Molecular Cell Biology, 3:1552-1561 (1983).. Cells were plated at a density of  $3-4 \times 10^4$  cells/cm<sup>2</sup> onto Primaria plates (Falcon Co.) in hormonally defined media supplemented with 10% fetal bovine serum; 4-6 hours later the media was replaced with fresh hormonally defined media which was subsequently changed every 24 hours during the duration of the experiment. Enat, R. et al., Proceedings of the National Academy of Sciences, USA, 81:1411-1415 (1984).

Hepatocyte cultures were infected for 12 hours with viral stocks (5 ml/10 cm plate) containing Polybrene (8 ug/ml). Unconcentrated viral stocks were prepared from the producer cells as described above.

DNA and RNA Analysis. High-molecular-weight genomic DNA was isolated and analyzed for integration of proviral sequences. Total cellular RNA was prepared using a guanidine thiocyanate procedure, fractionated in formaldehyde/agarose gells and transferred to

-43-

- nitrocellulose paper. Chirgwin, J.M. et al., Biochemistry, 18:5294-5299 (1980). Northern and Southern blots were probed with a 1.9 kb LDLR cDNA fragment (Hind III to Eco RI fragment of pTZ1) that was labeled to high specific activity with <sup>32</sup>P-dCTP using the random primer method. Feinberg, A.P. and B. Vogelstein, Anal. Biochem., 132:6-13 (1984). Northern blots were stripped and reprobed with a cDNA probe for human gamma actin (Hind III to Bam HI fragment of pHF-1). Gunning, P. et al., Molecular Cell Biology, 3:787-795 (1983).
- Cytochemical Analyses. Hepatocytes cultures infected with the BAG virus were analyzed for expression of viral directed beta-galactosidase using a cytochemical stain that forms a blue precipitate in the cytoplasm of transduced cells. Price, J. et al., Proceedings of the National Academy of Sciences, USA, 84:156-160 (1987). Glucose-6-phosphatase activity was detected by the lead phosphate enzyme cytochemical procedure. Wachstein, M. and E. Meisel, J. Histochem. Cytochem., 4:592 (1956).
- Cultures were analyzed for the presence of LDLR or the receptor for acetylated LDL (AcLDL) by incubating the cultures in hormonally defined media containing fluorescent labeled LDL or AcLDL (labeled with 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate - hereafter abbreviated as Dil - at 10 ug/ml, and obtained from Biomedical Tech. Inc., Stoughton, MA) for 6-8 hours, followed by three rinses with phosphate buffered saline and fixation in phosphate buffered saline containing 0.5% gluteraldehyde. Pitos, R.E. et al., Arteriosclerosis, 1:177-185 (1981). Voyta, J.C. et al., Journal of Cellular Biology, 99:81A (1984). Uptake of

-44-

the fluorescinated reagents was visualized in situ using an inverted Leitz fluorescent microscope.

05     Assay of LDL Degradation. Five-day-old cultures of hepatocytes plated in 35 mm dishes were assayed for degradation of  $^{125}\text{I}$ -LDL (10 ug/ml, 0.15 uCi/ug, obtained from Biomedical Tech., Inc., Stoughton, MA) using the procedure described by Goldstein, Basu and Brown. Goldstein, J.L. et al., Methods in Enzymology, 98:241-260 (1983).

10     Generation of Recombinant Retroviruses Encoding Human LDLR

Four different retroviral vectors were tested; the proviral components of these vectors are presented in Figure 3. Each vector differs in the transcriptional elements used to drive the expression of LDLR: LTR-LDLR - viral long terminal repeat sequences (LTR); BA-LDLR contains a 267 bp segment of the chicken beta-actin gene (BA) extending from -266 to +1. H4-LDLR contains a 704 bp segment of the histone H4 gene (H4) extending from -696 to +8. TK-LDLR contains a 256 bp segment of the thymidine kinase gene of herpes simplex virus (TK), extending from -200 to +56. Plasmid sequences of LTR-LDLR were derived from the 7.2 Kb Bam HI to Cla I fragment of DC1 with the following modification:

25     sequences spanning the Nhe I to Xba I sites of the 3' Moloney murine leukemia virus (Mo-MLV) LTR (nuc. 7846 to 8113) were replaced with homologous sequences from the LTR of the myeloproliferative sarcoma virus (represented by darkened area). Korman, A.J. et al., Proceedings of the National Academy of Sciences, USA, 84:2150-2154 (1987); Van Beveren, C. et al., In: RNA Tumor Viruses (2nd edition), Weiss, R. et al. (ed.), Cold Spring Harbor

30

-45-

Laboratory, pp. 766-783 (1985); Stacey, A. et al.,  
Journal of Virology, 50:725-732 (1984).

05 The backbone structure of plasmids BA-LDLR, H4-LDLR,  
and TK-LDLR (including the 5'-LTR, flanking mouse genomic  
DNA, pBR322 sequences, and 3'-LTR with contiguous pro-  
viral sequence to the Cla I site at nucleotide 7674) was  
derived from D01, with the exception that sequences  
containing the viral enhanced elements of the 3' LTR  
(from the Pvu II site at nucleotide 7933 to the Xba I  
10 site at nucleotide 8111) were deleted (indicated by the  
inverted triangle). This was done to reduce the amount  
of viral transcription after reverse transcription and  
integration of the recombinant provirus. These vectors  
also contained additional Mo-MLV sequence between the 5'  
15 LTR and the internal promoters. The additional sequence  
was derived from wild type Mo-MLV (from nucleotide 146 at  
the border of U5 to the Xho I site in the gag coding  
region at nucleotide 1560) with the exception that a Sac  
II linker was inserted at the Hae III site at nucleotide  
20 624. (This additional sequence is noted as gag in the  
figure). In each case LDLR coding sequences were derived  
from a 2.6 kb Hind III fragment of plasmid pTZ1 which  
contains a full-length LDLR cDNA insert (kindly provided  
by Drs. D. Russell, J. Goldstein and M. Brown). S.D.  
25 indicates splice donor site; arrows under each vector  
show the sites of transcriptional initiation.

Virus producing cell lines for the vector BA-LDLR,  
H4-LDLR, and TK-LDLR were made by transfecting the  
particular plasmid DNA with pSV2-Neo into the amphotropic  
30 packaging cell line Psi-Crip, as described previously.  
Cone, R.D. et al., Molecular and Cellular Biology, 7:887-  
897 (1987); Mulligan, R.C. and P. Berg, Science,



-46-

209:1422-1427 (1980). Psi-Crip is a modified type of Psi-am packaging cell line which provides cells with the functions of Psi-am (e.g., gag, pol, and env) in two components. That is, the gag-pol function is provided in  
05 the form of one integrated provirus and the env function is produced from a separate provirus. The host range is amphotropic.

G418 resistant colonies of cells were expanded and tested for production of virus that transmitted the  
10 correct proviral structure. This was done by harvesting supernatants from the producer cells, infecting NIH3T3 cells, and analyzing the infected population for integrated provirus by Southern analysis.

High titer amphotropic producers of the LTR-LDLR  
15 vector were obtained using a 2-step procedure. First, high titer ecotropic producers were made by cotransfection of LTR-LDLR with pSV2-Neo into the Psi-2 packaging cell line as described above. Mann, R. et al., Cell,  
33:153-159 (1983). Psi-Crip cells were then infected  
20 with virus harvested from the Psi-2 producer and subsequently split into 10 cm plates at clonal densities. Individual clones were isolated and analyzed for the production of high titer amphotropic virus as described above. Virus-producing cell lines transmitting the  
25 highest number of proviral copies to recipient cells were chosen for this study. All virus producing cell lines were maintained in culture for 4-6 weeks prior to their use in order to test for the presence of helper virus. None of the cell lines yielded any detectable helper  
30 virus nor transferred the packaging functions. A NIH3T3 cell line, designated 7-35, producing amphotropic virus that expresses the gene for human LDL receptor has been

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deposited (February 3, 1988) under the terms of the Budapest Treaty, with the American Type Culture Collection (Rockville, MD) under accession number CRL 9635.

05        Transfer and Expression of LDLR in Hepatocytes

Cells used for the infection studies were hepatocytes isolated from 3 NZW rabbits and 4 WHHL rabbits as described above. Collagenase perfusions routinely produced  $40-80 \times 10^6$  cells/animal with greater than 90% viability. Cells plated at subconfluent densities formed aggregates (5-20 cells/aggregate) that covered approximately 20% of the dish when visualized 6 hours after plating. The primary cultures underwent marked proliferation after 36 hours in culture achieving confluence by day 3 or 4.

To document the cellular composition of the cultures, mock infected WHHL hepatocytes cultured for 5 days were analyzed in several ways. First, the cells were stained for glucose-6-phosphatase, as described above, to determine the number of hepatocytes in the cultures. Glucose-6-phosphatase is a specific marker for hepatocytes in sections of liver and in hepatocyte cultures. More than 95% of the cells had the brown cytoplasmic staining characteristic of hepatocytes. No staining was detectable in pure cultures of fibroblasts or endothelial cells.

In addition, the cultures were analyzed for the presence of endothelial cells and Kupffer cells, since these nonparenchymal cells are abundant in the intact liver and could potentially contaminate the primary cultures. Sinusoidal and capillary endothelial cells as well as Kupffer cells express high levels of the receptor

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for AcLDL and can be identified in mixed cultures by their selective uptake of Dil-AcLDL. Analysis of the cultured hepatocytes for Dil-AcLDL uptake revealed that approximately 1 in 50 cells were fluorescent. The uptake  
05 of Dil-AcLDL by the rare contaminating cells in the hepatocyte cultures was equivalent to that observed in pure secondary cultures of endothelial cells derived from bovine aorta.

To optimize the conditions for infection of the  
10 hepatocytes, a high titer amphotropic virus (BAG) encoding E. coli beta-galactosidase was used. Cells transduced by the BAG virus can be detected by a simple cytochemical reaction that stains the cell's cytoplasm blue. Optimal transduction of WHHL hepatocytes was  
15 achieved when the cells were plated at subconfluent density and exposed to virus 36 hours after the initial plating. The matrix substrata had little effect on transduction efficiency.

Having optimized the conditions for infection, NZW  
20 and WHHL hepatocytes were infected with the four different LDLR virus preparations 2 days after being placed in culture and were analyzed for gene transfer and LDLR expression on day 5. Integration of the recombinant proviral sequences into the cellular DNA isolated from  
25 infected hepatocytes was detected by Southern blot analysis (Figure 6). DNA from transduced hepatocytes was digested with Kpn I and analyzed by the method of Southern using the LDLR cDNA as a probe. Kpn I has  
30 unique recognition sites in the LTR sequences; consequently, each integrated provirus should yield a common restriction fragment irrespective of the site of integration. Each virus producing cell line efficiently

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transmitted proviral sequences without rearrangement in hepatocytes from both NZW and WHHL rabbits. The relative copy number of integrated provirus varied from a maximum of 1-2 copies/cell for cultures infected with the H4-LDLR virus to a minimum of 0.1 to 0.2 copies/cell for cultures infected with the LTR-LDLR virus. This efficiency of infection was approximately 50% of that achieved in murine fibroblastic cells infected with the same virus preparations.

Additional experiments were performed to show that the viral DNA detected in Figure 6 was integrated into hepatocyte DNA. DNAs from transduced hepatocytes were digested with Eco RI (a restriction enzyme that has a single site in the proviral DNA) and subjected to Southern analysis using an LDLR probe. If the viral DNA existed as an integrated provirus no distinct Eco RI fragments should be detected because the outer borders of these fragments are located in flanking DNA and therefore are heterogenous. In fact, no Eco RI fragments were detected when this analysis was done, suggesting that the majority of viral DNA was integrated into hepatocyte chromosomal DNA.

Transduced cultures were first analyzed for LDLR expression by Northern analysis (Figure 7). A faint band with an apparent molecular size equal to 3.5 Kb was detected in mock infected cultures (Figure 7). This band, which probably represents endogenous LDLR RNA, was consistently more intense in WHHL cultures than in NZW cultures. The predominant RNA species in cultures infected with BA-LDLR, H4-LDLR, and TK-LDLR were the transcripts initiated at the internal promoter. The relative abundance of these RNAs consistently varied in

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vector-dependent manner as follows: BA-LDLR H4-LDLR  
TK-LDLR putative endogenous signal. As expected, very  
little transcription initiated from the LTR of these  
vectors was detected since the enhancer deletion which is  
05 present in the 3' LTR of the starting plasmid is  
transferred to the 5' LTR during proviral passage into  
hepatocytes. Cultures infected with the LTR-LDLR virus  
produced a single very intense band representing a  
transcript initiated at the LTR. All blots were stripped  
10 and reprobated with a human gamma-action cDNA probe to  
control for variation in the amount of RNA that was  
loaded. There was no detectable variation in the  
intensity of the gamma-action band suggesting that equal  
quantities of undegraded RNA were loaded.

15 Biochemical activity of the exogenous LDLR was  
assessed in situ by visualizing the uptake of LDL;  
transduced cultures of hepatocytes were incubated with  
Dil-LDL and viewed by fluorescent microscopy. Mock  
infected NZW rabbits exhibited a uniformly high level of  
20 fluorescence in all cells; mock infected WHHL hepatocytes  
showed very little fluorescence. WHHL hepatocytes  
infected with the LTR-LDLR virus had the greatest amount  
of LDL uptake with approximately 20% of the cells showing  
high levels of fluorescence. BA-LDLR infected WHHL  
25 hepatocytes demonstrated a population of cells with  
moderate activity; H4-LDLR infected WHHL hepatocytes  
showed a low level of activity in virtually all cells.  
The activity of LDLR in TK-LDLR infected cells was barely  
over background. The estimate of transduction efficiency  
30 based on this in situ assay for LDLR activity agrees with  
that measured by Southern analysis (e.g., WHHL  
hepatocytes infected with the LTR-LDLR virus showed

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fluorescence in approximately 20% of cells while Southern analysis detected a copy number of integrated proviral sequences equal to approximately 0.2).

Transduced hepatocytes were also analyzed for  
05 degradation of  $^{125}\text{I}$ -LDL in an attempt to quantify the  
amount of human LDLR expressed. These data are summarized in the Table. Activity of LDLR was greatest in  
hepatocytes infected with the LTR-LDLR virus: hepatocytes from a NZW rabbit showed an increase in LDLR  
10 activity from 170 ng/mg/5hrs in mock infected cells to  
274 ng/mg/5hrs in transduced cells, while cells from WHHL  
rabbits exhibited an increase in activity from 30-40  
ng/mg/5hrs in mock infected cells to 155 (WHHL 1) and 84  
(WHHL 3) ng/mg/5hrs in transduced cells. The level of  
15 LDLR activity in LTR-LDLR transduced hepatocytes is  
approximately 700 ng/mg/5hrs (4 fold greater than the  
activity of the endogenous receptor in NZW rabbits) when  
corrected for the actual number of cells that were  
transduced. Dzierzak, E.A. et al., Nature, 331:35-41  
20 (1987). Hepatocytes infected with viruses that express  
LDLR from a transcript driven by an internal promoter  
(i.e., BA-LDLR, H4-LDLR, and TK-LDLR) exhibited little to  
modest increases in  $^{125}\text{I}$ -LDL degradation.

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Table Quantitative analysis of  $^{125}\text{I}$ -LDL degradation in transduced hepatocytes<sup>1</sup>

|              |          | <u><math>^{125}\text{I}</math>-LDL Degradation (ng/mg protein/5h)<sup>2</sup></u> |                  |                         |
|--------------|----------|---|------------------|-------------------------|
| <u>Virus</u> |          | <u>NZW</u>  | <u>WHHL-1</u>    | <u>WHHL-3</u>           |
| 05.          | mock     | 170 $\pm$ 8 <sup>3</sup>  | --               | 38 $\pm$ 5 <sup>3</sup> |
|              | BA-LDLR  | 201 $\pm$ 10  | --               | 42 $\pm$ 3              |
|              | H4-LDLR  | 194 $\pm$ 9   | 30 <sup>4</sup>  | 47 $\pm$ 3              |
|              | TK-LDLR  | 188 $\pm$ 17  | --               | 44 $\pm$ 8              |
|              | LTR-LDLR | 274 $\pm$ 6   | 155 <sup>4</sup> | 84 $\pm$ 13             |

10 <sup>1</sup>Degradation rates were also measured in the presence of 50 fold excess of unlabeled LDL. Under these conditions NZW hepatocytes (mock infected and transduced) had degradation rates that ranged from 50 to 60 ng/mg/5 hrs while WHHL hepatocytes had degradation rates that ranged from 10 to 20 ng/mg/5 hrs.

15 <sup>2</sup>Analyses were performed on selected cultures of one NZW rabbit and two WHHL rabbits (WHHL 1 and WHHL 3).

<sup>3</sup>Represents mean  $\pm$ 1 S.D. (N=3 for WHHL 3 and N=4 for NZW).

<sup>4</sup>Single determinations.

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EXAMPLE V Introduction of Genetically Modified  
Hepatocytes into Rabbits

The technique described by Demetriou et al. was used to develop methods to restore LDL receptor function in the WHHL rabbit. Demetriou, A.A. et al., Science, 233: 1190-1192 (1986). Briefly, allogenic hepatocytes with normal LDL receptor function were isolated from New Zealand white (NZW) rabbits by collagenase perfusion. The isolated hepatocytes were bound to collagen coated dextran microcarrier beads and injected intraperitoneally (IP) into WHHL rabbits ( $2 \times 10^8$  cells/ rabbits). As a control for nonspecific effects of the intervention on serum cholesterol, hepatocytes from outbred WHHL rabbits bound to microcarrier beads were injected IP into another group of WHHL rabbits.

Serum was subsequently obtained and analyzed at various times for serum cholesterol levels. In the group of WHHL rabbits injected with NZW hepatocytes, serum cholesterol progressively declined by  $25 \pm 4\%$  (N=4) over 3 to 4 days, and subsequently increased to baseline levels by day 9. In the control group, serum cholesterol levels actually increased transiently to  $114 \pm 4\%$  (N=2) of control and returned to baseline levels by day 7.

A second set of similar experiments was performed in which the hepatocytes from the NZW rabbits were injected into the portal veins of WHHL rabbits. The serum cholesterol levels progressively declined by  $25 \pm 4\%$  (N=3) for four days after injection and subsequently increased to baseline levels by day 11.

In another experiment, hepatocytes from a WHHL rabbit were isolated by collagenase perfusion for in vivo



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studies. The hepatocytes were plated as primary cultures, infected with either the LTR-LDLR recombinant retrovirus or mock infected (See Example IV). The hepatocytes were harvested and injected into the portal vein of WHHL rabbits. The animal transplanted with mock-infected cells demonstrated a transient increase in cholesterol to a  $115 \pm 6\%$  ( $N=2$ ), while the animal transplanted with LTR-LDL infected cells demonstrated a decrease in serum cholesterol by 30% over a 3 day period.

10 EXAMPLE VI Expression of human parathyroid hormone by  
rat hepatocytes

Hepatocytes were isolated from a Wistar rat, as described in Example I. Cells were plated at a density of  $10 \times 10^6$  cells onto a bacteriologic plate (10 cm) with 160 mg of cytodex beads and inoculated with hormonally defined medium (HDM) with 10% fetal calf serum. After 90 minutes, the media was replaced with fresh HDM. On day three, the plate was inoculated with viral supernatant from an amphotropic PTH producer (prepared as described in Example II). After 12 hours, the cells were transplanted into an analbuminemic rat (approximately 200 gm intraperitoneally in approximately 5 ml. phosphate buffered saline).

Serum was subsequently obtained and analyzed at various times (see Figure 8) for human PTH, using the commercially-available Nichols radioimmunoassay procedure. Serum was analyzed for rat albumin using Western blot analysis. Results are presented in Figure 8.

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Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

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CLAIMS

1. Transduced hepatocytes, comprising incorporated genetic material.
- 05 2. Genetically modified hepatocytes of Claim 1 wherein the incorporated genetic material is genetic material of interest comprising:
  - 10 a. genetic material present in and expressed at biologically effective levels by normal hepatocytes, but present in or expressed in less than normal quantities in the hepatocytes prior to stable transfer of genetic material of interest into them;
  - b. genetic material not present in normal hepatocytes; or
  - 15 c. genetic material present in normal hepatocytes but not expressed at biologically effective levels in such cells, alone or in any combination thereof.
- 20 3. Transduced hepatocytes of Claim 2, additionally comprising genetic material encoding a selectable marker.
4. Hepatocytes infected with a viral vector which has a recombinant genome, the recombinant genome comprising incorporated genetic material.
- 25 5. Hepatocytes of Claim 4 in which the incorporated genetic material is:

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- 05      a.    genetic material present in and expressed at  
         biologically effective levels by normal hepato-  
         cytes, but present in or expressed in less than  
         normal quantities in the hepatocytes prior to  
         stable transfer of genetic material of interest  
         into them;
- b.    genetic material not present in normal hepato-  
         cytes; or
- 10      c.    genetic material present in normal hepatocytes  
         but not expressed at biologically effective  
         levels in such cells, alone or in any combina-  
         tion thereof.
- 15      6.    Hepatocytes of Claim 5, wherein the incorporated  
         genetic material additionally comprises genetic  
         material encoding at least one selectable marker.
7.    Hepatocytes of Claim 6 in which at least one select-  
         able marker is a gene encoding antibiotic resistance  
         or a gene that complements a genetic defect in the  
         host species.
- 20      8.    Hepatocytes of Claim 7 in which the gene encoding  
         antibiotic resistance is the neo gene and the gene  
         encoding an essential product is the his D gene.
9.    Human hepatocytes of Claim 5 in which the incorpo-  
         rated genetic material encodes a hormone, an enzyme  
25      or a drug.

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10. Human hepatocytes capable of expressing incorporated genetic material, the incorporated genetic material selected from the group consisting of:
  - 05 a. genetic material present in and expressed at biologically effective levels by normal hepatocytes, but present in or expressed in less than normal quantities in the hepatocytes prior to stable transfer of genetic material of interest into them;
  - 10 b. genetic material not present in normal hepatocytes; or
  - 15 c. genetic material present in normal hepatocytes but not expressed at biologically effective levels in such cells, alone or in any combination thereof.
11. Hepatocytes having incorporated therein recombinant amphotropic retrovirus having a recombinant genome comprised of:
  - 20 a. long terminal repeat sequences, the tRNA binding site and the Psi packing site derived from amphotropic Moloney murine leukemia virus; and
  - b. genetic material encoding at least one polypeptide of interest.
12. Hepatocytes of Claim 11 in which the recombinant genome additionally comprises genetic material encoding at least one selectable marker.
- 25 13. Hepatocytes of Claim 13 in which the genetic material encoding at least one selectable marker is a neo gene.

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14. Hepatocytes of Claim 14 in which the genetic material encodes human low density lipoprotein receptor.
- 05 15. Transduced hepatocytes capable of episomal expression of incorporated genetic material.
16. A method of making transduced hepatocytes comprising incorporated genetic material which encodes at least one polypeptide of interest, comprising:
  - 10 a. contacting cultured hepatocytes with media containing an infectious recombinant retrovirus having a recombinant genome comprised of genetic material of interest; and
  - 15 b. maintaining the cultured hepatocytes and the media containing the infectious recombinant retrovirus under conditions appropriate for infection of the hepatocytes by recombinant retrovirus.
17. A method of Claim 16 in which the incorporated genetic material additionally comprises genetic material encoding at least one dominant selectable  
20 marker.
18. A method of making hepatocytes, capable of expressing incorporated genetic material encoding at least one protein or at least one polypeptide of interest, comprising:
  - 25 a. transducing cultured hepatocytes with genetic material encoding at least one protein or at least one polypeptide of interest and

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- b. culturing transduced hepatocytes under conditions appropriate for their growth.
19. A method of implanting genetically modified hepatocytes which express incorporated genetic material of interest in a mammal, comprising the steps of:
- a. contacting cultured hepatocytes with medium containing an infectious recombinant retrovirus having a recombinant genome comprised of genetic material of interest;
  - b. maintaining the cultured hepatocytes and the medium under conditions appropriate for infection of the hepatocytes by recombinant retrovirus; and
  - c. introducing the modified hepatocytes produced in (b) into the mammal
20. A method of implanting genetically transduced hepatocytes capable of expressing incorporated genetic material of interest in a mammal, comprising the steps of:
- a. transducing cultured hepatocytes with genetic material encoding at least one protein or at least one polypeptide of interest;
  - b. culturing transduced hepatocytes under conditions appropriate for their growth; and
  - c. introducing the transduced hepatocytes produced in (b) into the mammal.
21. A method of treating a defect in liver function which is the result of abnormal production of a

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protein or a polypeptide, comprising introducing into the liver transduced hepatocytes comprising genetic material encoding the protein or the polypeptide whose abnormal production in the hepatocyte is the cause of the defect, under conditions appropriate for expression of the protein or the polypeptide in the hepatocytes.

22. A method of providing a hormone, an enzyme or a drug to an individual, comprising transducing hepatocytes with genetic material encoding the hormone, the enzyme or the drug and introducing the transduced hepatocytes into the individual.

23. A method of providing a protein or a polypeptide normally expressed by hepatocytes to an individual in whom expression of the protein or the polypeptide is compromised, comprising:

a. transducing hepatocytes with a recombinant retrovirus having a recombinant genome comprised of:

- 1) genetic material encoding the protein or the polypeptide;
- 2) the long terminal repeat sequences, the tRNA binding site and the Psi packaging site derived from an amphotropic retrovirus; and
- 3) at least one promoter of eukaryotic origin; and

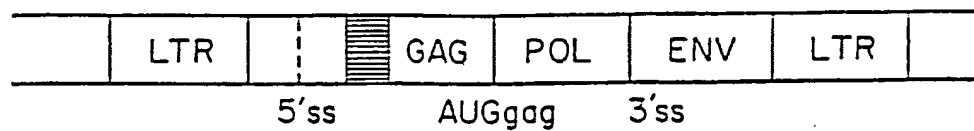
b. introducing transduced hepatocytes into the individual.



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24. A method of Claim 23 in which the recombinant genome is additionally comprised of a promoter of eukaryotic origin which can be modulated by an external cue.
- 05 25. A method of Claim 24 in which the recombinant genome is additionally comprised of genetic material encoding at least one dominant selectable marker.
26. A method of Claim 25 in which at least one dominant selectable marker is a gene encoding antibiotic resistance or a gene that complements a genetic defect in the host species.
- 10 27. A recombinant vector for transducing hepatocytes with genetic material encoding a protein of interest or a polypeptide of interest.

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*Fig. 1*

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Fig. 2a

pLJ

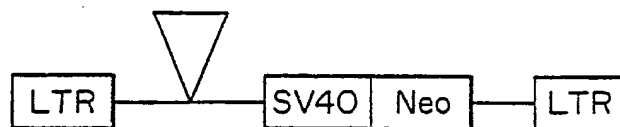


Fig. 2b

pEm

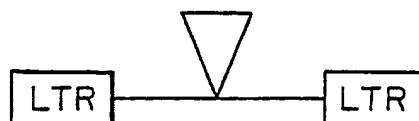


Fig. 2c

pIp



PRO =  $\beta$ -Actin  
Histone  
Thymidine kinase  
Thy-1  
Albumin

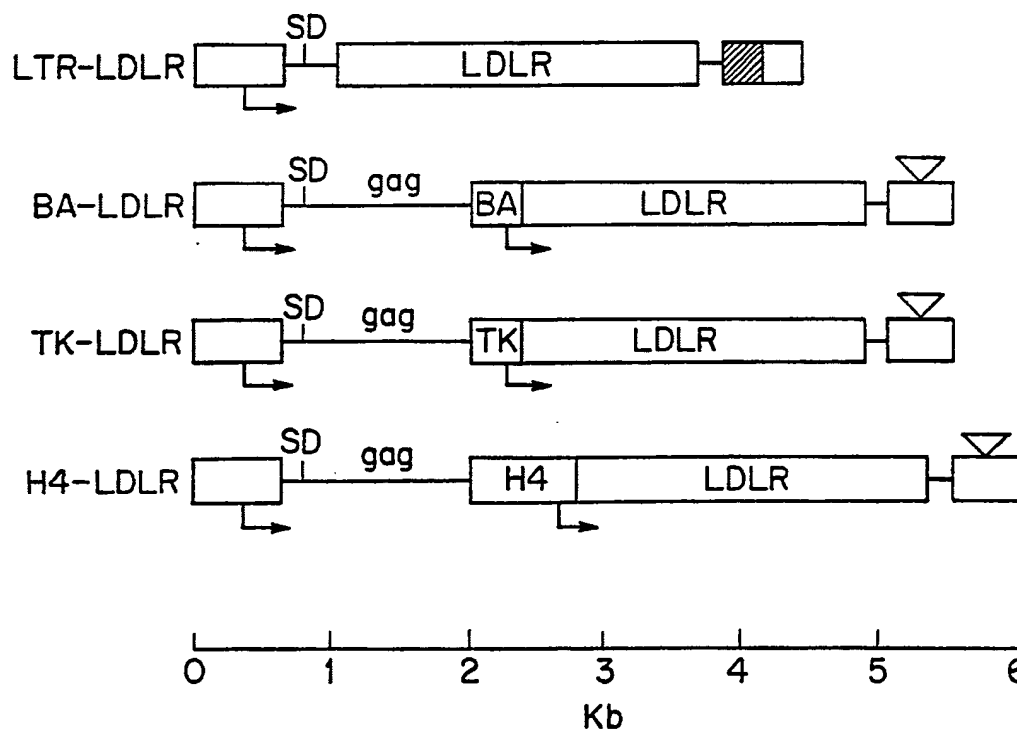


Fig. 3

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FIG. 4 A.

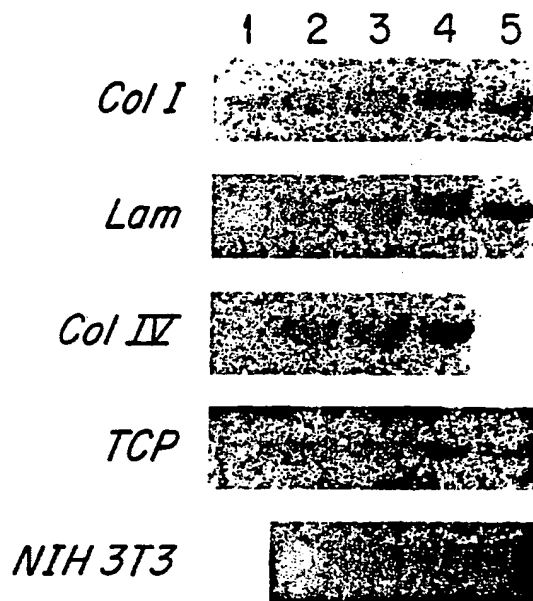


FIG. 4 B.

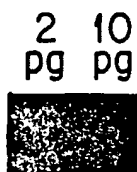


FIG.5a      FIG.5b      FIG.5c

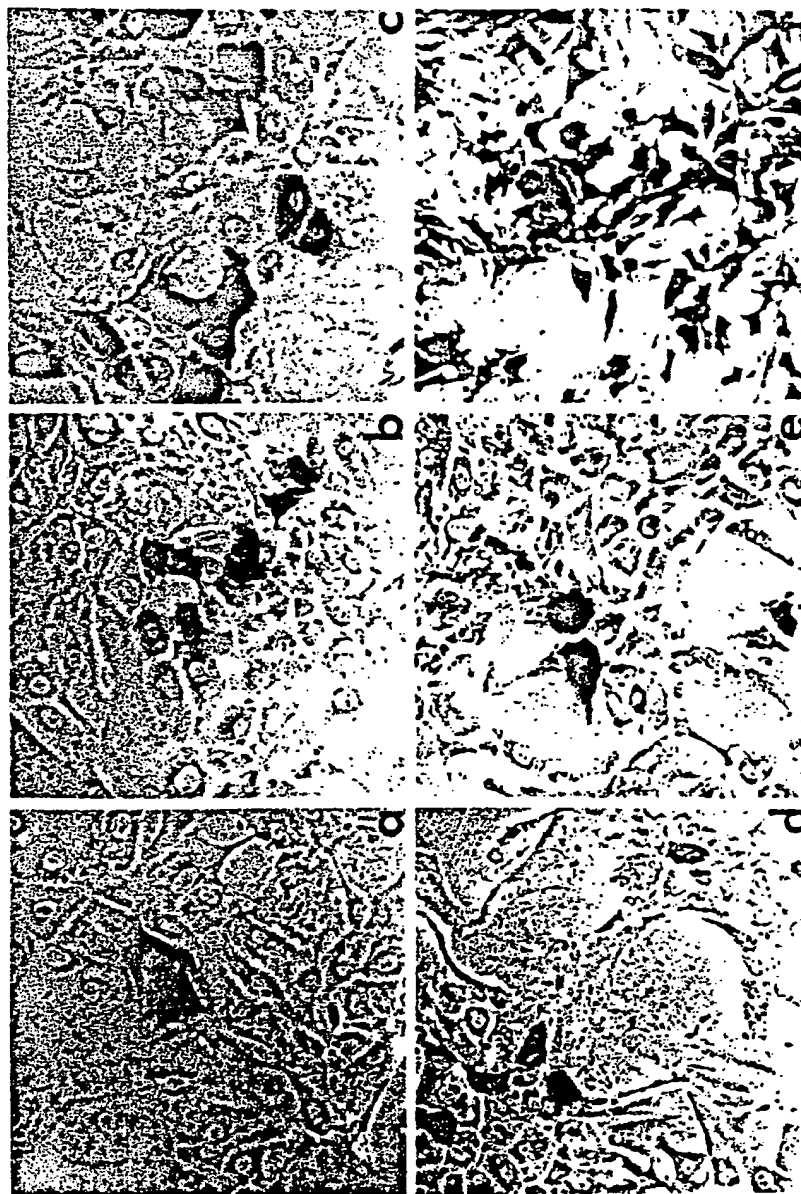


FIG.5d      FIG.5e      FIG.5f

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FIG.5i



FIG.5h



FIG.5g



FIG.5k

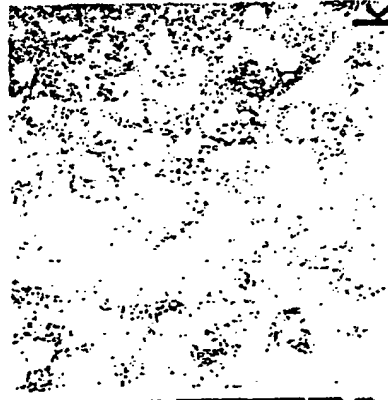
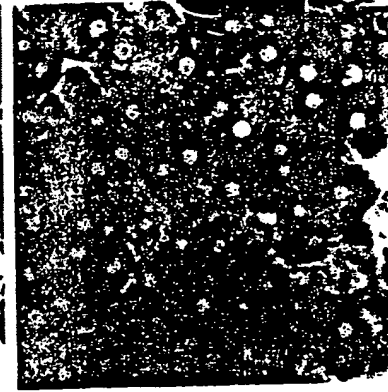


FIG.5j



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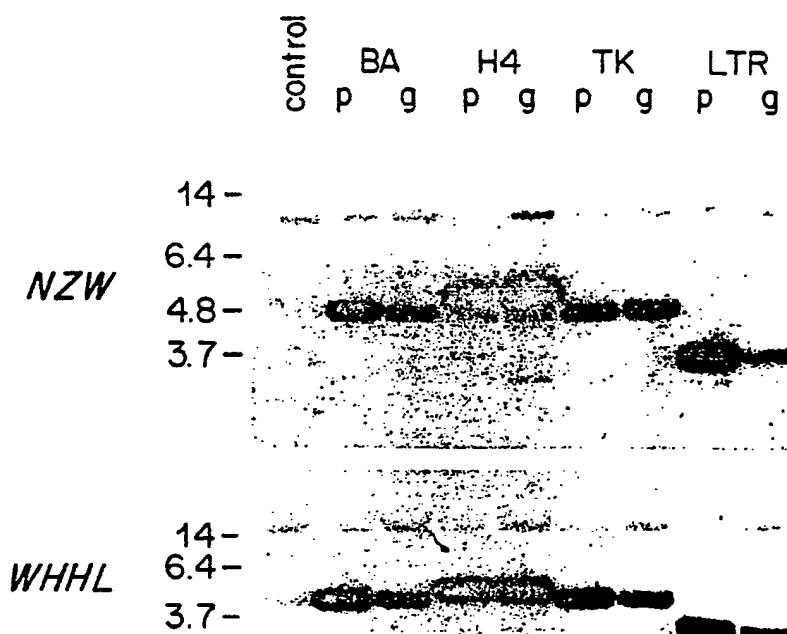


FIG. 6

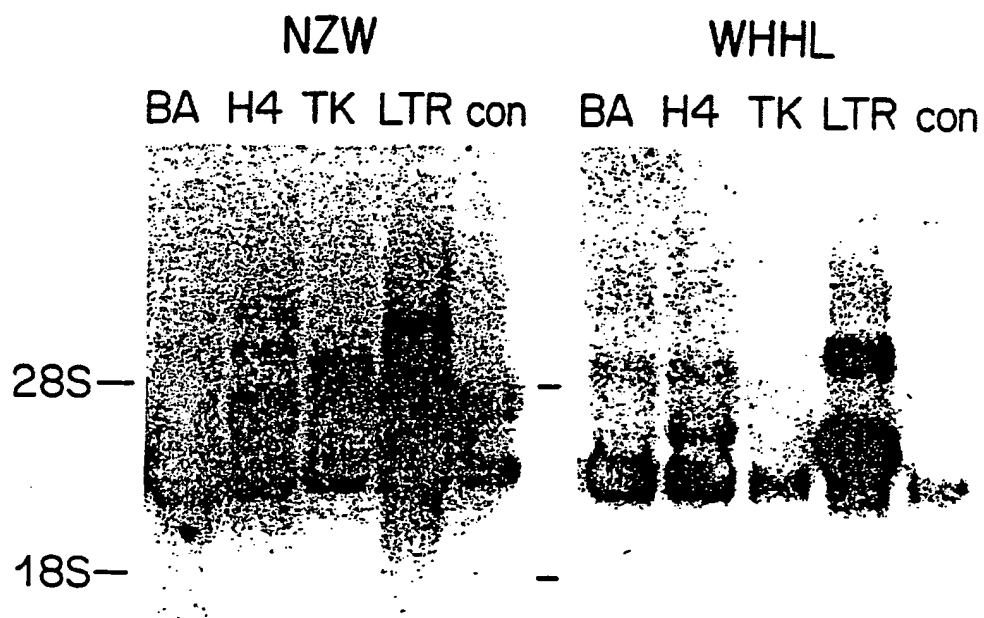


FIG. 7

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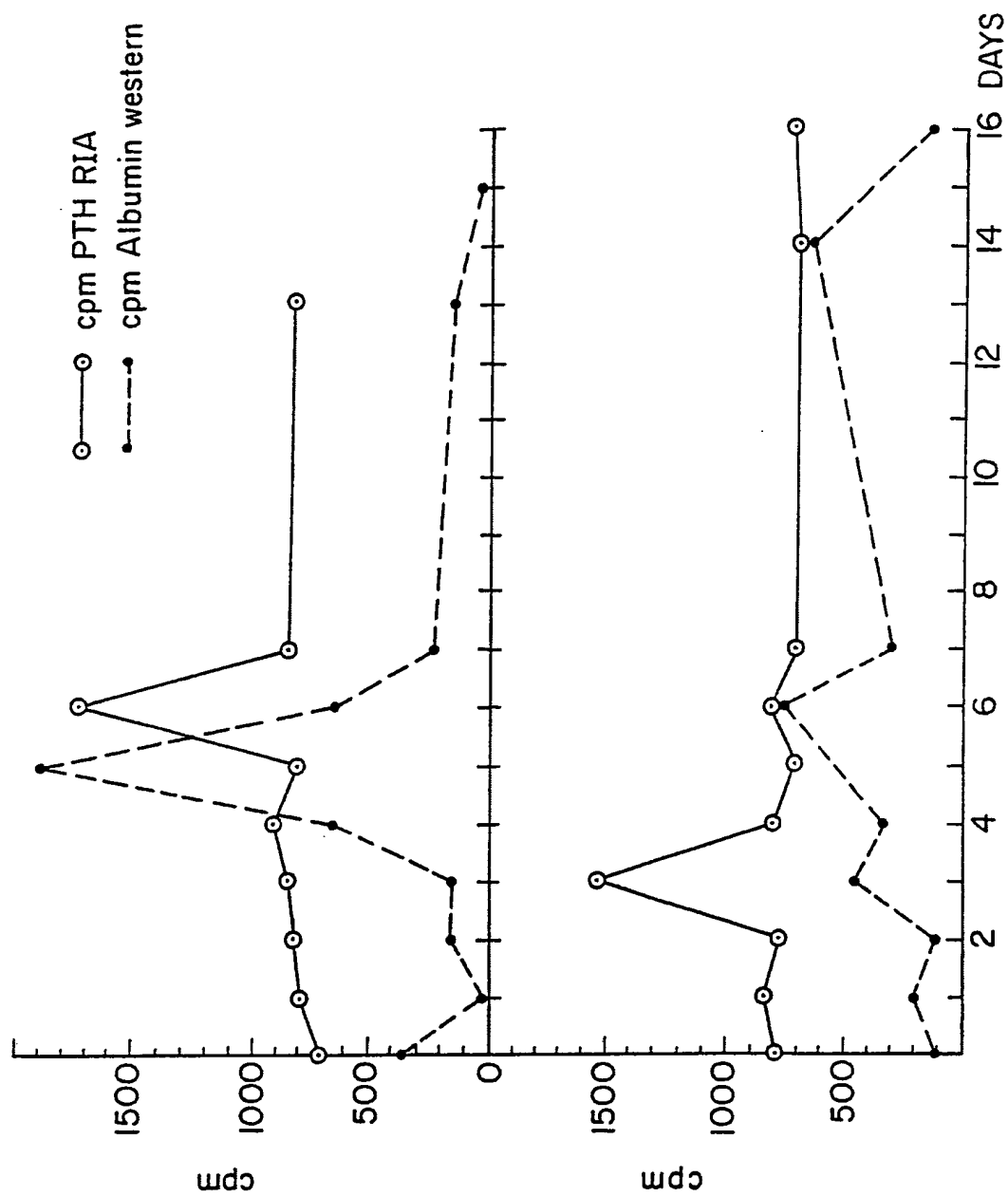


Fig. 8





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |  |   |
|--|--|---|
| (51) International Patent Classification <sup>4</sup> :<br><br>C12N 5/00, 15/00  | A3   | (11) International Publication Number: WO 89/ 07136<br>(43) International Publication Date: 10 August 1989 (10.08.89) |
| (21) International Application Number: PCT/US89/00422<br>(22) International Filing Date: 2 February 1989 (02.02.89)<br>(31) Priority Application Number: 152,749<br>(32) Priority Date: 5 February 1988 (05.02.88)<br>(33) Priority Country: US<br>(71) Applicants: WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US). HOWARD HUGHES MEDICAL INSTITUTE [US/US]; 6701 Rockledge Drive, Bethesda, MD 20817 (US).<br>(72) Inventors: WILSON, James, M. ; 24 Whitman Road, Waltham, MA 02154 (US). MULLIGAN, Richard, C. ; 441 Franklin Street, Cambridge, MA 02138 (US). | (74) Agents: GRANAHAN, Patricia et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).<br>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).<br>Published<br><i>With international search report.<br/>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i><br>(88) Date of publication of the international search report: 24 August 1989 (24.08.89) |   |
| (54) Title: MODIFIED HEPATOCYTES AND USES THEREFOR   |  |   |
| <p style="text-align: center;">Kb</p>  |  |   |
| (57) Abstract<br>Genetically engineered or transduced hepatocytes which express genetic material of interest introduced or incorporated into them, as well as methods of producing, transplanting and using the genetically engineered hepatocytes. The genetic material of interest can be incorporated through use of a vector, such as a recombinant retrovirus, which contains the genetic material of interest, or by other means.  |  |   |


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# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/00422

|  |  |  |
|--|--|--|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>  |  |  |
| According to International Patent Classification (IPC) or to both National Classification and IPC  |  |  |
| IPC <sup>4</sup> : C 12 N 5/00; C 12 N 15/00   |  |  |
| <b>II. FIELDS SEARCHED</b>   |  |  |
| Minimum Documentation Searched <sup>7</sup>  |  |  |
| Classification System  | Classification Symbols   |  |
| IPC <sup>4</sup>   | C 12 N   |  |
| Documentation Searched other than Minimum Documentation<br>to the extent that such Documents are Included in the Fields Searched <sup>8</sup>  |  |  |
|  |  |  |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> <sup>9</sup>   |  |  |
| Category <sup>10</sup>   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>   | Relevant to Claim No. <sup>13</sup>  |
| X  | Proc. Natl. Acad. Sci. USA, vol. 84,<br>May 1987,<br>J.A. Wolff et al.: "Expression of<br>retrovirally transduced genes in<br>primary cultures of adult rat<br>hepatocytes", pages 3344-3348<br>see the whole article<br>--  | 1-8,11-13,<br>15-18,27   |
| X  | Proc. Natl. Acad. Sci. USA, vol. 81,<br>October 1984,<br>H.C. Isom et al.: "Quantitative<br>assay for albumin-producing liver<br>cells after simian virus 40<br>transformation of rat hepatocytes<br>maintained in chemically defined<br>medium", pages 6378-6382<br>see the whole article<br>-- | 1-6  |
| ./.  |  |  |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div> |  |  |
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| Date of the Actual Completion of the International Search  |  | Date of Mailing of this International Search Report  |
| 19th May 1989  |  | 25. 07. 89   |
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| EUROPEAN PATENT OFFICE   |  |  P.C.G. VAN DER PIJPEN |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

|     |   |                  |
|-----|---|------------------|
| X,P | Proc. Natl. Acad. Sci. USA, vol. 85,<br>June 1988,<br>J.M. Wilson et al.: "Correction of<br>the genetic defect in hepatocytes<br>from the Watanabe heritable<br>hyperlipidemic rabbit", pages 4421-4425<br>see the whole article<br>----- | 1-8,11-18,<br>27 |
|-----|---|------------------|

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 19-26, because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1 (iv)  
Methods for treatment of the human or animal body by  
surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(e).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

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- ☐ The additional search fees were accompanied by applicant's protest.  
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